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Ex Vivo Stability of the Rodent-Borne Hantaan Virus in Comparison to That of Arthropod-Borne Members of the *Bunyaviridae* Family▽

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The possible effect of virus adaptation to different transmission routes on virus stability in the environment is not well known. In this study we have compared the stabilities of three viruses within the *Bunyaviridae* family: the rodent-borne *Hantavirus* Hantaan virus (HTNV), the sand fly-borne *Phlebovirus* sandfly fever Sicilian virus (SFSV), and the tick-borne *Nairovirus* Crimean-Congo hemorrhagic fever virus (CCHFV). These viruses differ in their transmission routes: SFSV and CCHFV are vector borne, whereas HTNV is spread directly between its hosts, and to humans, via the environment. We studied whether these viruses differed regarding stability when kept outside of the host. Viral survival was analyzed at different time points upon exposure to different temperatures (4°C, 20°C, and 37°C) and drying at 20°C. We observed clearly different stabilities under wet conditions, particularly at 4°C, where infectious SFSV, HTNV, and CCHFV were detectable after 528, 96, and 15 days, respectively. All three viruses were equally sensitive to drying, as shown by drying on aluminum discs. Furthermore, HTNV and SFSV partially survived for 2 min in 30% ethanol, whereas CCHFV did not. Electron microscopy images of HTNV, SSFSV, and CCHFV stored at 37°C until infectivity was lost still showed the occurrence of virions, but with abnormal shapes and densities compared to those of the nonincubated samples. In conclusion, our study points out important differences in ex vivo stability among viruses within the *Bunyaviridae* family.

Members of the *Bunyaviridae* family are widely spread around the world and can cause severe and often even fatal zoonotic diseases. The *Bunyaviridae* family consists of five genera (*Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus*, and *Tospovirus*). The viruses are morphologically similar, with a spherical structure and a lipid envelope. Each virion is 80 to 120 nm in diameter and contains a tripartite negative-sense-RNA genome (22). However, the natural hosts and transmission cycles differ. Most viruses in the *Bunyaviridae* family are carried by arthropods and are transmitted to humans or other mammals during blood feeding; therefore, these viruses are unlikely to be exposed to an environment outside the host. Hantaviruses, on the other hand, are rodent borne. The transmission between rodents and from rodents to humans occurs through wounding or by inhalation of aerosolized virus-contaminated excreta in the environment (21). We recently showed that hantaviruses can survive and be transmitted to other rodents for up to 15 days after being excreted (11). In theory, for transmission, hantaviruses would therefore benefit from an improved stability outside the host, while this would be of less importance for the vector-borne members of the same family.

Hantaan virus (HTNV), the prototype virus within the *Hantavirus* genus, is found in Asia and causes hemorrhagic fever with renal syndrome in humans, with a case fatality index of approximately 10%. It is spread by the striped field mouse *Apodemus agrarius* (15). Sandfly fever Sicilian virus (SFSV) is spread by *Phlebotomus papatasi* sand flies in southern Europe and is maintained by transovarial transmission. SFSV can infect humans via blood-feeding sand flies and causes relatively mild symptoms, such as fever, headache, and muscle and joint pain (3). The low viral titers in infected vertebrates, together with the finding that sand flies are refractory to laboratory oral infections, make an arthropod-vertebrate-arthropod circulation less probable (24). Crimean-Congo hemorrhagic fever virus (CCHFV) is transmitted and maintained by ixodid ticks and spread to several vertebral species in parts of Europe, Africa, and Asia. CCHFV causes a lethal disease with hemorrhagic manifestations in humans (26). CCHFV can also be transmitted to humans via direct contact with infected patients or by contact with blood or tissues from viremic livestock (7).

In this study we compared the stabilities of a rodent-borne *Hantavirus* (HTNV), a sand fly-borne *Phlebovirus* (SFSV), and a tick-borne *Nairovirus* (CCHFV) in a wet environment at different temperatures and under dry conditions. For laboratory safety reasons, we also compared the susceptibilities to inactivation in ethanol.

MATERIALS AND METHODS

Viruses and cell cultures. The viruses used in this study were cell line-adapted HTNV (strain 76-118) (3.5×10^6 focus-forming units/ml), SFSV (strain Sabin) (8.2×10^4 PFU/ml), and CCHFV (strain IbAr 10200) (4×10^4 focus-forming units/ml). All viruses were grown in Vero E6 cells with minimal essential medium (Gibco, Paisley, Scotland) supplemented with 2 mM L-glutamine (Sigma, Steinheim, Germany), 100 U/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma), 0.3% bicarbonate (Sigma), and 5% fetal bovine serum (Sigma). The virus samples used for the stability tests were stored at −80°C in vials containing 0.5 ml of cell-free virus supernatant. All experiments with HTNV and SFSV were carried out in a biosafety level 3 laboratory, whereas CCHFV was handled in a biosafety level 4 facility at the Swedish Institute for Infectious Disease Control.

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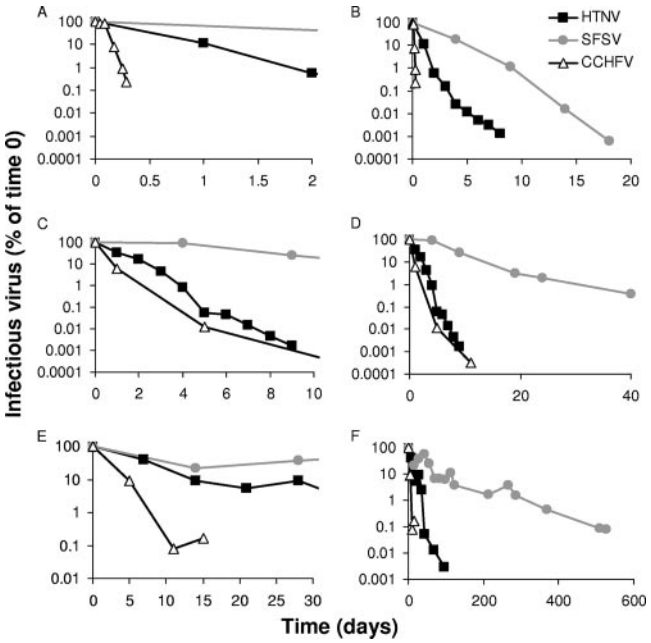

FIG. 1. Virus stability in medium. Viruses were thawed and incubated at various temperatures (A and B, 37°C; C and D, 20°C; E and F, 4°C). Infectivity was measured by titration on Vero E6 cells and is indicated as the percentage of input virus concentration detected at time zero. Early time points are shown in more detail in panels A, C, and E. Time points represent titrations of single vials from the same virus stock.

Determination of virus concentrations. By carrying out the different stability tests described below, virus concentration was determined as follows. For HTNV, virus suspensions of unknown concentrations were diluted 10-fold in dilution medium (Hanks' balanced salt solution [Gibco] supplemented with 2% HEPES [Gibco], 2% fetal bovine serum, 100 U/ml penicillin [Sigma], and 100 μg/ml streptomycin [Sigma]) and titrated as described earlier (12). Briefly, 200 μl of each dilution was incubated in duplicate for 1 h on confluent Vero E6 cells, grown in 24-well cell culture plates, and subsequently overlaid with 0.5% agarose medium and incubated for 7 days before being fixed and developed. Foci of infected cells were visualized and counted using polyclonal rabbit anti-Dobrava hantavirus or anti-Saaremaa hantavirus serum, followed by horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G and 3,3′,5,5′-tetramethylbenzidine.

For SFSV, concentrations were determined by a plaque assay as described earlier (6, 18). Briefly, virus was treated as described above with the exception that Vero E6 cells, after infection, were covered with an agar overlay (50% modified Eagle medium [2×] [Gibco], 2% DEAE-dextran, 1% dimethyl sulfoxide [Sigma], 1% agar [Becton Dickinson and Company, Sparks, MD], and 0.2% heparin [LEO Pharma AB, Malmö, Sweden] in sterile water). After 4 days, a second layer of agar mixture, containing 60 μg/ml neutral red, was added. Plaques were counted at day 5 after infection.

For CCHFV, concentrations were determined mainly as described earlier (1). Briefly, Vero E6 cells grown in 96-well plates were infected by 10-fold serial dilutions of CCHFV. Cells were fixed in a mixture of acetone-methanol at 24 h postinfection and stained with rabbit anti-CCHFV nucleocapsid protein antibodies for 40 min followed by fluorescein isothiocyanate-conjugated anti-rabbit antibodies for 30 min. Foci were counted using a fluorescence microscope (Nikon Eclipse TE300).

The experimental sensitivities for the detection of infectious viruses were 5 infectious viruses/ml for HTNV and SFSV and 10 infectious viruses/ml for CCHFV.

Stability tests. The two different environmental conditions used in the experiments were (i) storage in cell-free medium at different temperatures and (ii) drying on a metal surface. One experiment was also designed to test virus inactivation in ethanol.

Wet conditions. After thawing, the virus-containing vials were placed in the dark at different temperatures (4°C, 20°C, and 37°C) and left for different periods of time before titrations were carried out as described above.

Dry conditions. Testing under dry conditions was performed by use of a modified version of an earlier-described method (9). Drops of 5-μl virus suspension were placed on sterilized aluminum discs (10-mm diameter) and left to dry in a biosafety cabinet at 20°C. At time points of 30, 60, and 90 min, the discs were transferred to 0.5 ml of dilution medium and vortexed to maximize virus elution in the medium. To measure the virus titer before drying, virus was eluted immediately after application to the discs. The eluted virus was titrated as described above.

Inactivation in ethanol. In order to test virus inactivation in ethanol, HTNV, SFSV, and CCHFV were added to different concentrations of ethanol in dilution medium to a final volume of 1 ml. The solutions were vortexed and incubated for 2 min at 20°C before being titrated as described above. A certain amount of ethanol was unavoidably left in the virus suspensions during titration and inoculation on cells. This small amount of ethanol was equal for all treated viruses.

Electron microscopy. Studies with SFSV and HTNV (nonincubated or incubated at 37°C for 35 days) and CCHFV (nonincubated or incubated at 37°C for 3 days) were performed using a Philips CM100 electron microscope (Eindhoven, The Netherlands). After fixation in 1.25% glutaraldehyde, virus particles were pelleted on carbon/Formvar-coated 400-mesh copper grids (GilderGrids, Lincolnshire, England). Briefly, 150 μl of virus suspension was centrifuged for 10 min in an Eppendorf 5417C centrifuge (Hamburg, Germany) with a swing-out rotor at a maximum force of 12,000 × *g*. The grids were placed on the flat bottom of the outer container of Sarstedt microvette CB 300 tubes (Nümbrecht-Rommelsdorf, Germany). Ten grid squares were counted in each case. One particle per square equals a concentration of 1.5×10^5 particles per ml. Grids were stained by 2% tungstophosphoric acid (Merck, Darmstadt, Germany) at pH 6.

RESULTS

Wet conditions. As expected, under wet conditions, a strong correlation between temperature and stability was observed: the higher the temperature, the shorter the survival of the viruses. Clear differences in stability were observed among the three viruses at all investigated temperatures. Generally, CCHFV was found to be the least stable virus whereas SFSV remained infectious for the longest time periods at all temperatures.

At 37°C, HTNV, SFSV, and CCHFV remained infectious for at least 8 days, 18 days, and 7 h, respectively (Fig. 1A and B and Table 1). At 20°C, these time points were extended to 9, 40, and 11 days (Fig. 1C and D and Table 1). At 4°C, the viruses remained infectious for at least 96, 528, and 15 days (Fig. 1E and F and Table 1).

Dry conditions. The drops of virus solutions dried within 25 min (data not shown). Infectious particles, representing 6 to 14% of the input concentration, were extracted from the three assayed viruses after 90 min (Fig. 2). There was no significant difference among the amounts of viable viruses at the different time points ($P > 0.05$, Kruskal-Wallis test). We were not able to detect any infectious virus from any of the genera from discs incubated for 24 h or longer (data not shown).

TABLE 1. Latest time points for detection of infectious virus under wet conditions

Virus	Latest time point virus detected at:		
	37°C	20°C	4°C
HTNV	8 days	9 days	96 days
SFSV	18 days	40 days	528 days
CCHFV	7 h	11 days	15 days

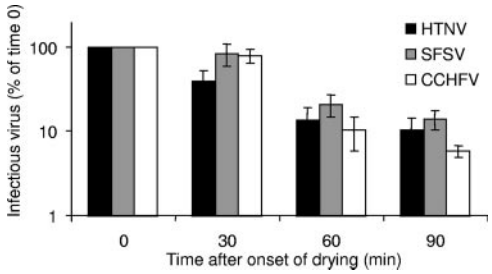

FIG. 2. Survival of virus dried on aluminum discs. Viruses were thawed, applied on sterile aluminum discs, and left to dry. After 0, 30, 60, and 90 min, the virus was eluted in medium and infectivity measured by titration on Vero E6 cells (indicated as percentage of input concentration detected at time zero). There was no significant difference among the viruses at any time point ($P > 0.05$, Kruskal-Wallis test). Each bar represents the mean ± standard deviation determined by three separate experiments.

Inactivation in ethanol. HTNV, SFSV, and CCHFV were undetectable after 2 min in 40% ethanol whereas HTNV and SFSV were detected after 2 min in 30% ethanol. We were, however, unable to detect CCHFV after 2 min in 30% or 20% ethanol. A lower concentration (10%) of ethanol had no detectable effect on the inactivation of any of the viruses (Fig. 3).

Electron microscopy. Approximately the same concentrations of virus particles were found when comparing nonincubated (infectious) samples of HTNV, SFSV, and CCHFV to samples incubated in medium at 37°C (noninfectious). However, the appearances of the virions were different. The inner structure of the particles appeared to be denser in the nonincubated samples (Fig. 4, left column) than in the incubated samples with noninfectious virions (Fig. 4, right column). CCHFV most often appeared surrounded by a ring-like structure (Fig. 4, bottom row). Additional electron microscopy studies of SFSV, either nonincubated or incubated for 35 days at 37°C, showed aggregates consisting of 20 to 300 virions, with only few virions appearing alone (data not shown). The concentration of the observed aggregates in SFSV corresponded to the measured titer of the untreated SFSV stock according to the plaque assay. Extensive vortexing, as an attempt to disrupt the aggregates of SFSV, did not increase the infectivity titer (data not shown). CCHFV did sometimes appear in small aggregates consisting of a few virions (Fig. 4), but larger aggregates of up to about 300 particles were seen only for SFSV.

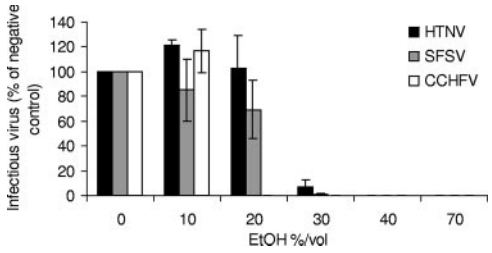

FIG. 3. Inactivation of virus in ethanol (EtOH). Viruses were diluted in different concentrations of ethanol and incubated for 2 min. Infectivity was measured by titration on Vero E6 cells and is indicated as the percentage of input concentration detected at time zero. Each bar represents the mean ± standard deviation determined by three separate experiments.

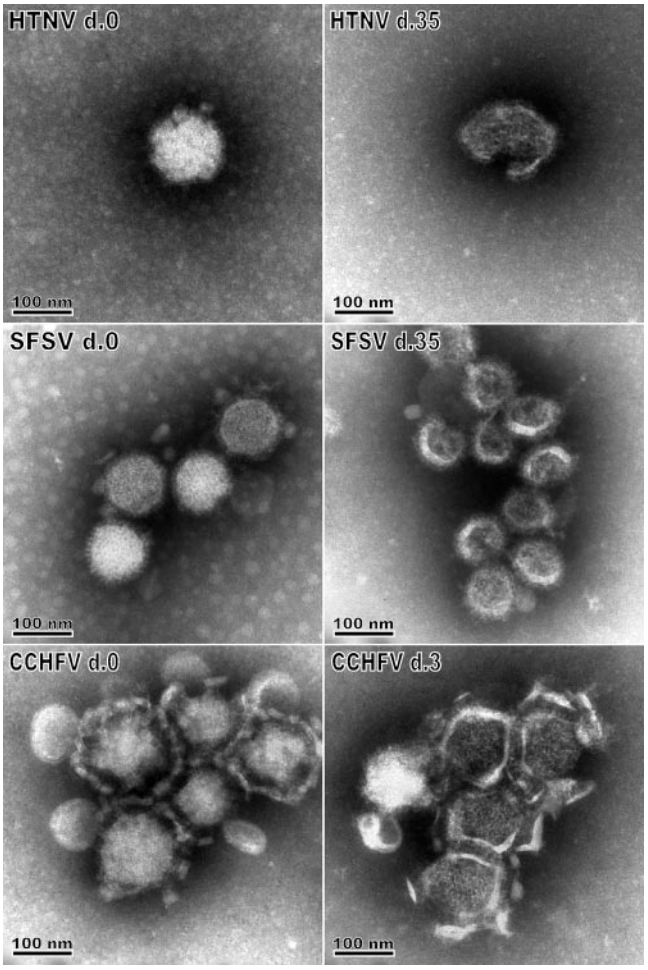

FIG. 4. Electron micrographs. Samples of HTNV, SFSV, and CCHFV were incubated at 37°C for the indicated time points (d.0, day 0; d.35, day 35; d.3, day 3) before being examined by electron microscopy. The left column illustrates nonincubated (infectious) samples, and the right column shows incubated samples with lost infectivity.

DISCUSSION

In order to increase the knowledge behind *Bunyaviridae* transmission and to obtain information useful when handling these highly pathogenic microorganisms, we have studied and compared survival outside the host for three viruses within this family.

We found that temperature had a large influence on the ex vivo stabilities of all three viruses. Interestingly, we also observed a clear difference in stability among the viruses. The sand fly-borne SFSV (*Phlebovirus*) was by far the most stable virus at all temperatures, which might reflect an adaptation to the exothermic host. In line with this, SFSV has rarely been found in mammals. Instead, vertical transmission seems to be most important for maintenance of the virus in nature (24). CCHFV, on the other hand, which is also transmitted by an exothermic host, was the least stable virus at all temperatures. Although transovarial transmission of CCHFV has been demonstrated for some vector species, the most important route for infection of ticks is probably via infected small vertebrates (7). Therefore, it could be speculated that the relatively short

survival of CCHFV in medium, shown in this study, might reflect that CCHFV is not adapted to surviving in an exothermic host. HTNV, the only virus in this study with a natural ex vivo occurrence, was not the most stable virus under wet conditions.

The importance of relative humidity for the survival of drying viruses has been shown by others (2, 10, 17). In the perspective of hantavirus ex vivo transmission in nature, it is interesting that all three viruses were equally resistant to drying. In an epidemiological study, a close association was found between the distribution of hantavirus-infected bank voles and wet habitats (25). It has also been reported that the seroprevalence for Puumala hantavirus (PUUV) among rodents trapped on north-facing slopes is higher than that among rodents trapped on south-facing slopes, suggested to be due to the higher humidity on the north-facing slopes (20). These findings indicate that, although transmitted ex vivo, hantaviruses do not seem to have evolved a stronger resistance to drying. Most likely, for extended ex vivo stability, hantaviruses are limited in their spread to high-humidity environments.

The stability of the viruses tested here is partially a result of the methods applied. Natural environments include propagation in cells of different origins as well as the presence of possible stabilizing substances. The difficulties associated with titration of wild-type hantavirus in cell culture (16) forced us to use cell line-adapted viruses in this study. Our group has previously examined the stability of wild-type PUUV in an intracage transmission study. Secreted PUUV remained viable and was able to infect other bank voles for up to 15 days (11). Although those experiments were somewhat different in setup, those results correlated well with results from our experiments on HTNV stability under wet conditions and at ambient temperature. Probably, the cage bedding offered a humid environment for efficient virus transmission.

PUUV has been found in oropharyngeal secretions, urine, and feces of its natural host, the bank vole (8, 28). In one of the studies, feces was shown to be the most common secretion to contain infectious PUUV (28). HTNV, on the other hand, is contained to a lesser extent in feces than in saliva or urine (14). One can speculate that rodent droppings provide a humid environment where the virus can remain in a certain state, but the importance of different excreta for spreading of hantaviruses remains to be shown clearly.

Generally, it is believed that nonenveloped viruses are more resistant to inactivation in ethanol; for instance, calicivirus retained 1 to 10% of its infectivity after 2 min in 70% ethanol (5). It has already been shown that HTNV is sensitive to a wide range of chemicals, including alcohol (13). We found that 70% ethanol completely inactivated all genera tested here, while both HTNV and SFSV were partially resistant to 30% ethanol. This observation is of importance since decontamination with ethanol is a commonly used method in the laboratory.

In order to find what caused the decreased infectivity over time, we performed electron microscopy of HTNV, SFSV, and CCHFV. We found approximately the same virus concentrations in nonincubated (infectious) and incubated samples where infectivity was lost. This, together with the abnormal shape and density of the virions in the incubated samples, indicates that inactivation is due to disruption of the virus particles. However, the detailed structural changes causing the decreased infectivity remain to be shown. The electron microscopy studies also revealed the presence of SFSV aggregates that corresponded well to the concentration of infectious units observed in our samples. This indicates that we, by use of the plaque assay, in fact were measuring the infection by these aggregates and not by individual virions. It was not possible for us to increase the virus titer by vortexing, but if an adequate method is found, the titers may increase approximately 100-fold, provided that all of the viruses within the aggregates are functional. Whether this aggregation phenomenon is beneficial in terms of increased stability or immune escape of the virus remains to be shown.

It is difficult to compare our findings to other reports, since we cannot exclude that the differences in stability arise from minor variations in experimental procedures. Furthermore, most studies of virus stability carried out under ex vivo conditions concern nonenveloped enteric viruses. Nevertheless, comparable survival data for CCHFV (4, 23) and for HTNV (27) have been reported earlier. Human immunodeficiency virus shows about 10% survival after 24 h at 25°C in phosphate-buffered saline (19), which is comparable to what was observed in our study. Many reports on virus survival concern only early time points. Since we are unaware of the infectious dose required for natural infection of the viruses studied in this report, it is pertinent to find the time point at which each virus has lost its infectivity completely.

In conclusion, viruses within the *Bunyaviridae* family, although similar in structure, show great variation in ex vivo stability under wet conditions, while they are equally sensitive to drying. Hantaviruses, although being non-vector borne, do not seem to have evolved a greater ability to survive ex vivo than CCHFV or SFSV.

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